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The Effect of Platelets in the Activation of Human Blood Coagulation Factor IX by Factor XIa

By Hans Soons, Truus Janssen-Claessen, H. Coenraad Hemker, and Guido Tans

We report here the effect of activated human platelets on the activation of human factor IX by human factor XIa. Factor IXa formed during activation was determined via its ability to activate bovine factor X. To increase sensitivity, phospholipids and bovine factor VIIIa were present in the assay. The kinetic parameters of the factor IX activation were determined in the presence of 10 mmol/L CaCl₂. The K_m for factor IX was 0.30 μ mol/L and k_{cat} was 2.4 s⁻¹. Activated human platelets inhibited factor IX activation by factor XIa in a dose-dependent manner, whereas unstimulated platelets had no effect. Factor IX activation was inhibited for more than 90% at a platelet concentration of 4×10^8 /mL, whereas concentrations of less than 10^8 /mL had no influence. The inhibitory effect could be induced by thrombin, collagen, calcium ionophore A 23187, and adrenalin. The appearance of inhibitory activity could be blocked by the addition of the prostacyclin analogue ZK 36374 at

any time during platelet activation. Stirring during platelet activation was not necessary. These results suggest that the inhibition is caused by a release reaction. This was confirmed by centrifugation experiments that showed that the inhibitory activity could be recovered from the supernatant of the activated platelets. The inhibitory activity was destroyed upon boiling and was susceptible to trypsin digestion. Passage of platelet supernatant over ACA 22 showed that the inhibitory activity eluted with an apparent molecular weight of less than 1,200,000 but greater than 669,000. The inhibition of factor XIa was reversible. These data suggest that platelets release an antiprotease of factor XIa that reversibly inhibits factor XIa. Lineweaver-Burk analysis showed that the inhibitor caused both an increase in K_m for factor IX and a decrease in k_{cat} of factor IXa formation by factor XIa.

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HUMAN FACTOR IX is a vitamin K-dependent plasma protein that participates in the intrinsic pathway of blood coagulation.¹ It is present in plasma as a zymogen with a molecular weight (mol wt) of 57,000 daltons (d) at a concentration of 2.6 to 5.0 μ g/mL. Human factor IX is converted to a serine protease, factor IXa, through proteolysis of two internal peptide bonds. This results in the release of an activation peptide (11,000 d) from the internal region of the precursor molecule. The resulting factor IXa (46,000 d) consists of a light chain (18,000 d) and heavy chain (28,000 d) held together by a disulfide bond(s).²⁻³ Activation of factor IX in plasma can be accomplished by factor XIa²⁻³ or by the factor VIIa-tissue thromboplastin complex.⁴⁻⁵

Platelets play an important role in blood coagulation. The platelet surface strongly promotes prothrombin and intrinsic factor X activation.⁶ The activation of factor IX by factor XIa is one of the few reactions of coagulation for which no surface requirement is known. It has been suggested that collagen-stimulated platelets exhibit factor XIa-like activity, which can be determined in a clotting test.⁷⁻⁸ However, others have failed to confirm this,⁹ and it is not known whether factor XIa is involved. Up till now nothing is known about whether platelets have any effect in the activation of factor IX by factor XIa.

Several assays have been described in literature for the determination of factor IX activation: a coagulation assay in factor IX-deficient plasma,¹⁰⁻¹² a tritiated activation peptide release assay,^{5,10,13} an immunoradiometric assay,¹¹ and an assay in which factor IXa is measured indirectly by determining the initial rate of factor X activation.¹⁴ The most

widespread used is the tritiated activation peptide release assay,^{5,10,13} which was originally described for the activation of factor X by Silverberg et al.¹⁵ In highly purified systems the appearance of factor IXa procoagulant activity follows the peptide release by a small lag period.¹⁰ However, in less-purified systems, other enzymes may be present that give rise to similar peptide fragments but destroy the procoagulant activity of factor IX.¹⁶ Therefore, to circumvent these potential difficulties, we have chosen to use an assay for factor IX activation by factor XIa, which detects the functional factor IXa activity via its ability to activate bovine factor X in the presence of phospholipids and CaCl₂.¹⁴ To increase its sensitivity, bovine factor VIIIa was present. In that case, however, care has to be taken to add factor VIIIa under rigorously standardized conditions, since the assay is critically dependent on the amounts of factor VIIIa present.

This study was undertaken to explore the role of platelets in factor IX activation by factor XIa. We present evidence that platelets, upon stimulation, secrete an inhibitor that interferes with factor IX activation by factor XIa.

MATERIALS AND METHODS

Materials. Chromogenic substrates L-pyroglyutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride (S2337) and *N*-benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-glycyl-L-arginine-*p*-nitroanilide hydrochloride S2366 were purchased from A.B. Kabi Diagnostica, Stockholm. The prostacyclin analogue ZK 36374 was a kind gift from Schering AG, Berlin. Plasma deficient in coagulation factors IX, XI, or XII were from patients congenitally deficient in these factors and were obtained from George King Biomedical, Overland Park, Kan. Hormon tendon collagen was obtained from Hormon-Chemi, Munich. Fatty acid-free human serum albumin and trypsin were from Sigma Chemical Co, St Louis. Two-milliliter plastic flat-bottom reaction tubes were obtained from Sterilin Ltd, Teddington, England; Teflon-coated magnetic stirring bars (7 \times 2 mm) were purchased from Bel-Art Products, Pequannock, NJ. All reagents used were of the highest grade commercially available.

Proteins. Human factor IX was purified based on a modification of the isolation procedure described by di Scipio et al.¹⁷ and the sulfated dextran column as described by Miletich et al.¹⁸ was added. Citrated fresh-frozen human plasma was thawed, and benzamidine

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(10 mmol/L), heparin (6 mg/ μ L), and soybean trypsin inhibitor (10 mg/L) were added. The barium citrate absorption was followed by ammonium sulfate precipitations (40% and 70% saturation) before the vitamin K-dependent protein preparation was applied to a diethyl aminoethyl (DEAE)-Sephadex column followed by a heparin-Sephadex column.¹⁷ Finally, a sulfated dextran column was used to purify factor IX.¹⁸ Human factor IXa was prepared from purified human factor IX as described earlier for bovine factor IXa.¹⁹ Human factor XI was purified according to Bouma et al.²⁰ Human factor XII was isolated as described by Griffin and Cochrane.²¹ β -Factor XIIa was prepared from the purified human factor XII as described by Fujikawa and McMullen.²² Bovine factor X was purified according to Fujikawa et al.²³ Bovine factor VIIIc was prepared as described earlier.²⁴ Factor VIIIa was prepared by activation of factor VIIIc (1.75 nmol/L) with thrombin (4 nmol/L) for one minute at 37 °C.^{19,24} Bovine thrombin was purified as a prothrombin activation product as described by Rosing et al.²⁵ The specific activity of the thrombin preparation was 1 National Institutes of Health (NIH) U/mL = 11 nmol/L when a molecular weight of 35,600 was used for thrombin. The human factor IX, XI, and XII and the bovine factor X₁ and X₂ preparations were homogeneous and pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate on 10% gels according to Laemmli.²⁶ The specific activities of the protein preparation as determined with a clotting assay were 193, 211, 74, and 100 U/mg for human factors IX, XI, XII, and bovine factor X, respectively, assuming one unit of human factor IX, XI, and XII to be present per milliliter of human normal plasma and one unit of bovine factor X in one milliliter of bovine normal plasma. All proteins were stored at -70 °C after dialyzing against the following buffers: 50 mmol/L Tris-HCl, 175 mmol/L NaCl at pH 7.9 for human factor IX and bovine factor X; 4 mmol/L sodium citrate, 2 mmol/L acetic acid, 0.15 mol/L NaCl, and 0.5 mmol/L EDTA at pH 5.0 for human factor XI and XII; and 10 mmol/L morpholino ethane sulfonic acid, 175 mmol/L NaCl, 10 mmol/L CaCl₂, and 10% glycerol at pH 6.5 for bovine factor VIII. Trypsin was insolubilized by coupling trypsin (60 mg) in 0.1 mol/L NaHCO₃ to CNBr-activated Sepharose 4B (3 g) at 4 °C overnight according to the manufacturer's instructions.

Preparation of human factor XIa. Human factor XIa was prepared from human factor XI using human β -factor XIIa.²⁷ To 10 mL of factor XI (0.15 mg/mL) in 4 mmol/L sodium acetate, 2 mmol/L acetic acid, 0.15 mol/L NaCl, pH 5.0, was added 1.25 mL 0.5 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 8.0, at 37 °C. Reaction was started by adding 1.27 mL β -factor XIIa (0.59 mg/mL) in 4 mmol/L sodium acetate, 2 mmol/L acetic acid, 0.15 mol/L NaCl at pH 5.0. Factor XI activation was followed by measuring factor XIa amidolytic activity. Activation was completed within 90 minutes. Factor XIa was separated from β -factor XIIa on a DEAE-Sephadex column (1.5 \times 11.5 cm) at 4 °C in 50 mmol/L Tris-HCl and 150 mmol/L NaCl at pH 8.0. Factor XIa did not adhere to the resin, whereas β -factor XIIa was eluted by a linear salt gradient (0.1 to 0.5 mol/L) NaCl.

The amidolytic activity of human factor XIa was measured using the chromogenic substrate S2366 in a buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 20 mmol/L EDTA, and 0.5 mg/mL human serum albumin, pH 7.9. The kinetic parameters of S2366 hydrolysis by human factor XIa were $K_m = 0.42$ mmol/L and $k_{cat} = 758$ s⁻¹. k_{cat} was calculated using a concentration of factor XIa based on a mol wt of 160,000.

Protein concentrations. Determinations of protein concentrations were routinely done according to Bradford.²⁸ Factors IXa, Xa and thrombin were determined by active-site titration with p-NPGB-p-nitrophenyl-p'-guanidinobenzoate (p-NPGB) as described earlier.^{14,25} Concentrations of factor IX and factor X were determined by complete activation and subsequent determination of

the active enzyme. Factor VIIIa concentrations were determined according to the method of van Dieijen et al.²⁴ Factor XIa concentrations were determined from the amount of protein present assuming a mol wt of 160,000 d.²⁵

Phospholipid vesicles. Phospholipid vesicles were made from a mixture of 40 mol/100 mL 1,2-dioleoyl-sn-glycero-3-phosphoserine (PS) and 60 mol/100 mL 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) in a buffer containing 50 mmol/L Tris-HCl and 175 mmol/L NaCl at pH 7.9 as described by Rosing et al.²⁵

Collagen. Collagen was added in the nonfibrillar form, dissolved in acidic dilution buffer obtained from Hormon-Chemie as described by Rosing et al.⁶ The minimal amount of collagen inducing aggregation and secretion was 1 to 2 μ g/mL.

Factor IXa determinations. To follow the activation of factor IX by factor XIa an assay system was developed in which factor IXa was determined via its ability to activate bovine factor X in the presence of CaCl₂, phospholipid, and factor VIIIa. Factor IXa-containing samples were diluted in ice-cold buffer containing 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 0.5 mg/mL human serum albumin, and 20 mmol/L EDTA to block further activation of factor IX. The amounts of factor IXa present in the samples were then determined as follows. Three hundred five microliters buffer containing 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 9.8 mmol/L CaCl₂, 0.5 mg/mL human serum albumin, and 16.4 μ mol/L phospholipid vesicles was prewarmed at 37 °C for 4½ minutes, after which 45 μ L bovine factor X (16.67 μ mol/L) and a 50- μ L sample were added. Factor X activation was started by adding 100 μ L factor VIIIa (1.75 nmol/L). The final reaction mixture (500 μ L) contained 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 6 mmol/L CaCl₂, 2 mmol/L EDTA, 10 μ mol/L phospholipid, 1.5 μ mol/L factor X, 0.35 nmol/L factor VIIIa, 0.5 mg/mL human serum albumin, and the factor IXa present in the sample. The rate of factor X activation was determined by measurement of the amounts of factor Xa formed after one and two minutes in these reaction mixtures using the factor Xa-specific chromogenic substrate S2337 as described earlier.^{14,19} From the observed rate of factor X activation, the amount of factor IXa present in the sample was calculated using a calibration curve made with known amounts of active-site titrated factor IXa (see also Fig 1).

Kinetic parameters. K_m for factor IX and k_{cat} of factor IX activation were determined by measurement of the rate of factor IXa formation in a reaction mixture containing 10 mmol/L CaCl₂, 0.5 mg/mL human serum albumin, 0.1 nmol/L factor XIa, and varying amounts of factor IX in HEPES buffer, pH 7.5, using the assay previously described. K_m and V_{max} (expressed as factor IXa formed per second) were then determined by statistical analysis of the data as described by Eisenthal and Cornish-Bowden.²⁹ k_{cat} was calculated by dividing V_{max} with the enzyme concentration assuming a mol wt of 160,000 for factor XIa.

Isolation of human platelets. Blood was drawn by venipuncture from healthy male volunteers who had not taken any medication for at least ten days. As anticoagulants, 1 vol of acid citrate dextrose (0.18 mol/L glucose, 0.08 mol/L trisodium citrate, and 0.052 mol/L citric acid) was added to 5 vol of whole blood. Platelet-rich plasma was obtained after centrifugation at 220 g for 25 minutes. Platelets were isolated by centrifugation at 1000 g for 20 minutes, and the pellet was gently resuspended in calcium-free HEPES buffer (10 mmol/L HEPES, 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl₂, and 5 mmol/L glucose), pH 6.7, containing 0.4% human serum albumin. The platelets were washed twice in this buffer by centrifugation at 600 g for 15 minutes. During the whole procedure except the last wash, acid/citrate/dextrose was present in the buffer (1 vol to 14 vol buffer).³⁰ Finally, the platelets were resuspended in HEPES buffer, pH 7.5, containing 0.1% human serum albumin. Platelet concentrations were determined with a Coulter counter

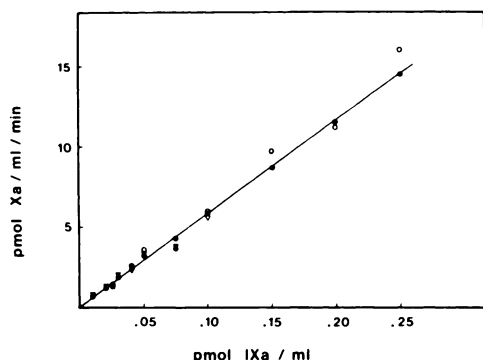


Fig 1. Factor IXa calibration curve. The rate of activation of bovine factor X (1.5 $\mu\text{mol/L}$) by varying amounts of factor IXa was determined in the absence (●) or in the presence of $3.2 \times 10^7/\text{mL}$ stimulated platelets (▽) or their supernatants (○) at 37°C in a reaction volume of 500 μL containing 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 6 mmol/L CaCl_2 , 2 mmol/L EDTA, 10 $\mu\text{mol/L}$ phospholipid vesicles (40% PS/60% PC, mole/mole), 1.5 $\mu\text{mol/L}$ factor X, 0.35 nmol/L factor VIIIa, 0.5 mg/mL human serum albumin, and amounts of factor IXa as indicated in the figure. When stimulated platelets or their supernatants were present, factor IXa was preincubated with the platelets or their supernatant for four minutes at 37°C prior to addition to the assay. Stimulated platelets were prepared by incubation at 37°C for 12 minutes with 1.2 nmol/L thrombin, 10 $\mu\text{g/mL}$ collagen, and 3 mmol/L CaCl_2 under continuous stirring. The supernatants were prepared by centrifuging the stimulated platelets for five minutes in an Eppendorf microfuge. For experimental details, see Materials and Methods under Factor IXa determination.

(Coulter Electronics, Luton, England) and were kept at room temperature.

RESULTS

Validation of the assay for factor IXa. In this study an assay for the activation of factor IX was used in which functional factor IXa activity was determined via the activation of bovine factor X as described earlier.¹⁴ To increase its sensitivity, the activation of bovine factor X by human factor IXa was performed in the presence of CaCl_2 , phospholipids, and bovine factor VIIIa. The amount of factor Xa formed was linear with time for at least two minutes (data not shown), and the rates of factor Xa formation were determined from the amounts of Factor Xa formed during these two minutes. Figure 1 shows that the rate of factor Xa formation was proportional to the amount of factor IXa added (closed circles). The slope of this plot reflects the rate of factor Xa formation per factor IXa present (59.5 nmol/Xa/min/nmol IXa). An excellent day-to-day reproducibility of the calibration curve could be achieved, since four separate experiments carried out at different days showed an SD of 1.7% in the slope of this plot.

This calibration curve was used in the experiments in which the activation of factor IX by factor XIa was determined. Since the experiments presented here concern the effect of platelets on the activation of factor IX by Factor XIa, the calibration curve was repeated in the presence of $3.2 \times 10^7/\text{mL}$ thrombin/collagen-stimulated platelets (Fig 1, open triangles) or the supernatant thereof (Fig 1, open circles). As can be seen, the presence of the triggered

platelets or their supernatants had no effect on the assay for factor IXa.

Factor IX activation by factor XIa. The assay just described was used to assess the kinetic parameters of factor IX activation by factor XIa in the presence of 10 mmol/L CaCl_2 . It was verified that factor IX activation was linear with time and proportional to the amount of factor XIa used (data not shown). Varying concentrations of factor IX were incubated with factor XIa, and the rate of factor IXa formation was determined in the assay just described. Michaelis-Menten kinetics were observed throughout. The kinetic parameters were $K_m = 301 \text{ nmol/L}$, and $k_{cat} = 2.4 \text{ s}^{-1}$ assuming a mol wt of 160,000 for factor XIa.

The effect of platelets in the activation of factor IX by factor XIa was studied. Nonstimulated platelets up to concentrations as high as $5 \times 10^8/\text{mL}$ had no effect on factor IX activation. However, in the presence of platelets stimulated by the combined action of thrombin and collagen, the rates of factor IXa formation decreased with time until after two minutes a low rate of factor IXa formation remained (Fig 2). Preincubation of factor IX with the activated platelets for four minutes did not change the time course of factor IX activation. However, when factor XIa was preincubated with

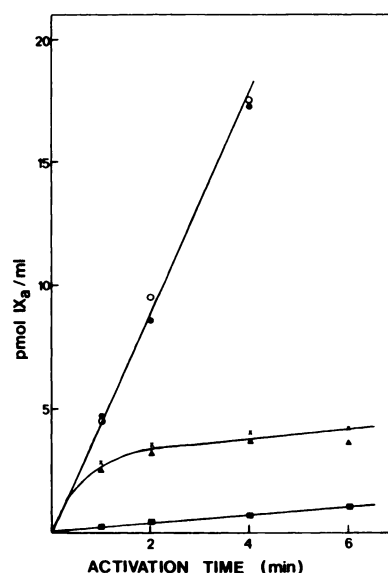


Fig 2. The effect of platelets in the activation of factor IX by factor XIa. The activation of factor IX (100 nmol/L) by factor XIa (0.1 nmol/L) was determined at 37°C in the absence of platelets (○), in the presence of $5 \times 10^8/\text{mL}$ unstimulated platelets (●), and in the presence of platelets ($5 \times 10^8/\text{mL}$) that had been stimulated for 12 minutes at 37°C under continuous stirring by thrombin (1.2 nmol/L), collagen (10 $\mu\text{g/mL}$), and 3 mmol/L CaCl_2 (X). The final reaction mixture contained 10 mmol/L CaCl_2 , 0.5 mg/mL human serum albumin, 100 nmol/L factor IX, 0.1 nmol/L factor XIa, and $5 \times 10^8/\text{mL}$ platelets in HEPES buffer at pH 7.5. At the time intervals indicated, samples were withdrawn and assayed for factor IXa as described in Materials and Methods. The points denoted (Δ) were obtained after factor IX was incubated four minutes with the stimulated platelets prior to the addition of factor XIa. The points denoted by (■) were obtained after a four-minute incubation of factor XIa with the stimulated platelets prior to the addition of factor IX.

the triggered platelets, only the low rate of factor IXa formation remained. This strongly suggests that the inhibition was the result of a direct interaction with factor XIa that was completed within four minutes. Subsequent experiments showed that for platelet concentrations ranging from 10^6 to 10^9 /mL a preincubation time with factor XIa of approximately ten minutes sufficed to result in linear rates of factor IXa formation. Therefore, in all subsequent experiments, factor XIa was preincubated for ten minutes with the platelets prior to the addition of factor IX.

The inhibitory effect of activated platelets at a suboptimal platelet concentration ($4 \cdot 10^7$ /mL) in factor IX activation by factor XIa was studied using different platelet agonists (Table 1). The most potent were thrombin (1.2 nmol/L), calcium ionophore A 23187 (1 μ mol/L) and the combined action of thrombin (1.2 nmol/L) and collagen (10 μ g/mL), whereas platelets activated by collagen (10 μ g/mL) or adrenalin (546 μ mol/L) caused a smaller inhibition. Platelets stimulated with adenosine diphosphate (ADP) (25 μ mol/L) whether in the absence or presence of fibrinogen (0.7 mg/mL), showed no significant inhibition of factor IX activation by factor XIa under these conditions. Stirring of the platelets was not necessary to achieve the inhibitory activity.

Figure 3 shows that factor IX activation was inhibited in a dose-dependent way. Factor IX formation was not influenced at platelet concentrations less than 10^6 /mL, and at higher platelet concentrations inhibition was observed until at a concentration of 4×10^8 /mL factor IX activation was almost completely inhibited. During the platelet activation the lysis was always smaller than 2%. Lactic dehydrogenase (LDH) was taken as the parameter for platelet lysis. One hundred

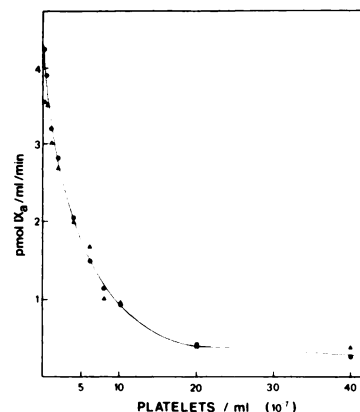


Fig 3. Factor IX activation in the presence of varying concentrations of activated platelets or their supernatants. Varying amounts of platelets were stimulated for 12 minutes at 37 °C under continuous stirring with thrombin (1.2 nmol/L) and collagen 10 μ g/mL in the presence of 3 mmol/L CaCl_2 . The platelets were then either incubated for an additional five minutes or centrifuged for five minutes in an Eppendorf Microfuge to obtain the supernatant. After this, the CaCl_2 concentration was adjusted to 10 mmol/L, and factor XIa was added and incubated for an additional ten minutes with the platelet suspensions or the supernatants. Subsequently, factor IX was added, and the rate of factor IXa formation was determined as described in Materials and Methods. The final reaction mixtures contained 10 mmol/L CaCl_2 , 0.5 mg/mL human serum albumin, 100 nmol/L factor IX, and 0.1 nmol/L factor XIa in HEPES buffer (pH 7.5) and amounts of platelets (●) or their supernatants (▲) as indicated in the figure.

Table 1. The Effect of Different Platelet Stimulators in Factor IX Activation by Factor XIa

Trigger Substance	Rate of Factor IX Activation
No trigger	100%
ADP	95%
ADP/fibrinogen	92%
Adrenalin	84%
Collagen	84%
Thrombin	62%
A23187	58%
Thrombin/collagen	59%
Thrombin/collagen*	58%

*The platelets were activated by thrombin (1.2 nmol/L) and collagen (10 μ g/mL) without stirring.

Platelets (4×10^7 /mL) were incubated in HEPES buffer (pH 7.5) at 37 °C for 12 minutes in the presence of ADP (25 μ mol/L), ADP (25 μ mol/L), human fibrinogen (0.7 mg/mL), adrenalin (546 μ mol/L), collagen (10 μ g/mL), ionophore A23187 (1 μ mol/L), thrombin (1.2 nmol/L), or thrombin (1.2 nmol/L), and collagen (10 μ g/mL). During stimulation the platelets were stirred. After this incubation, factor XIa was added and incubated for ten minutes with the activated platelet suspension. The factor IX activation mixture contained 4×10^7 /mL platelets, 100 nmol/L factor IX, 0.1 nmol/L factor XIa, and 10 mmol/L CaCl_2 in HEPES buffer, pH 7.5. Factor IX activation rates were determined as described in Materials and Methods and expressed as a percentage of the activation rate measured in the presence of unstimulated platelets (3.58 pmol IXa/min/mL).

percent lysis was the amount of LDH measured when the platelets were sonicated. When the triggered platelet suspension was centrifuged, all the inhibitory activity was recovered in the supernatant (Fig 3), indicating that the inhibition is the result of platelet secretion upon triggering.

The release of inhibition was studied using the prostacyclin analogue ZK 36374. This substance effectively blocks any further platelet aggregation³¹ and platelet release reactions³² when added to platelet suspensions (Fig 4). The addition of 100 nmol/L prostacyclin analogue prior to stimulation with 0.2 nmol/L thrombin effectively blocked any release of inhibitory activity. The time course of release of inhibitor was determined by the addition of ZK 36374 at various time intervals after the addition of thrombin to the platelets. The amounts of inhibitory activity released were then determined in the supernatant after centrifugation. Figure 5 shows that secretion of the inhibitor started after a small lag period of 10 seconds and reached a plateau after a platelet activation time of approximately four minutes.

Preliminary characterization of the inhibitory activity. To gain insight into the nature of the inhibitory activity secreted by platelets, the supernatant of a large amount of stimulated platelets (1.8×10^{10}) was collected, concentrated, and passed over a Sepharose ACA 22 column (2.5×85 cm). Figure 6 shows that the inhibitory activity was included in the column volume but eluted earlier than the largest mol wt marker available (669,000), thus precluding an accurate estimation of the apparent mol wt. The fractions of the column containing inhibitor were pooled and

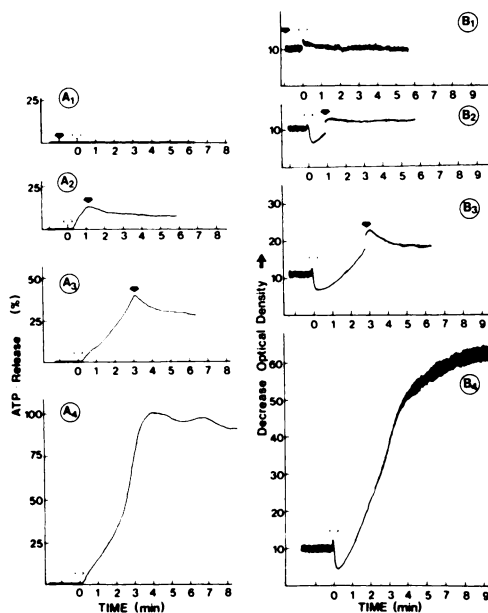


Fig 4. Influence of ZK 36374 on platelet aggregation and adenosine triphosphate (ATP) release. Platelet aggregation and ATP release were measured in an aggregometer. Washed platelets (2.3×10^8 /mL) in HEPES buffer containing 2.6 mmol/L CaCl_2 and luciferin luciferase reagents were placed in the aggregometer under stirring conditions. At time 0, platelet activation was started by adding (0.7 nmol/L, ie 0.06 U/mL) thrombin (open arrow). At different time points, ZK 36374 (90 nmol/L) was added to stop the platelet activation (closed arrow). ATP release was monitored by luciferin-luciferase reaction (A), and platelet aggregation was measured at the same time (B). When ZK 36374 was added one minute before thrombin, no ATP release and platelet aggregation was measured (A_1 and B_1). When ZK 36374 was added one or three minutes after thrombin, ATP release and aggregation stopped immediately (A_2 , B_2 , and A_3 , B_3). ATP release and platelet aggregation were also measured in the absence of ZK 36374 (A_4 and B_4). ATP release is expressed as a percentage, with ATP release in the absence of ZK 36374 taken as 100%.

concentrated, after which a part was treated with trypsin-Sepharose. Table 2 shows that no inhibitory activity remained after this treatment, whereas a control experiment with only Sepharose showed no loss of inhibitory activity. When the experiment with the trypsin-treated inhibitor was repeated in the absence of factor XIa, no activation of factor IX was observed, indicating that no significant amounts of trypsin were coming off the beads (data not shown). The inhibitor was also destroyed when placed in a boiling water bath for one hour. From these data we conclude that the inhibitor is a protein or contains a protein part essential for its activity that is susceptible to trypsin and is heat labile.

The data in Table 3 show that the inhibitor did not destroy or irreversibly inhibit factor XIa. When factor XIa was incubated with a high amount of inhibitor, only 21% of the rate of factor IX activation remained. However, when this incubation mixture was diluted fivefold, the rate of factor IX activation was only 1.6-fold less. Since rates of factor IX activation were linear with the amount of factor XIa present (see also the aforementioned), this indicated that some 50% of the factor XIa had become available again to participate

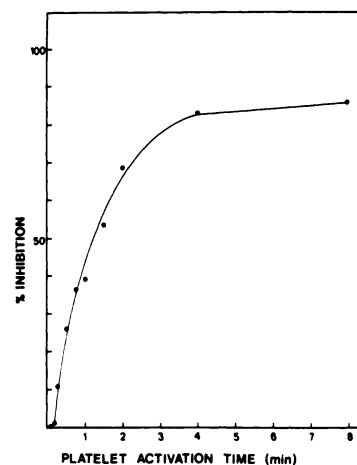


Fig 5. Time course of the appearance of inhibitory activity during platelet activation. Platelets (2.6×10^8 /mL) were stimulated at 37 °C with thrombin (0.2 nmol/L) in the presence of CaCl_2 (3 mmol/L) under continuous stirring. At the time points indicated, the release reaction was blocked by adding the prostacyclin analogue ZK 36374 (100 nmol/L), and the platelet suspension was centrifuged for two minutes in an Eppendorf Microfuge to obtain the supernatant. After this, the CaCl_2 concentration was adjusted to 10 mmol/L, and factor XIa was added and incubated with the supernatant for 20 minutes at 37 °C, after which factor IX activation was started with the addition of factor IX. The final activation mixture contained 10 mmol/L CaCl_2 , 0.5 mg/mL human serum albumin, 100 nmol/L factor IX, 0.1 nmol/L factor XIa, and supernatant of activated platelets (2×10^8 /mL) in HEPES buffer. The rate of factor IX activation in the reaction mixture was determined as described in Materials and Methods and was expressed as a percentage of the factor IX activation rate measured in the presence of unstimulated platelets.

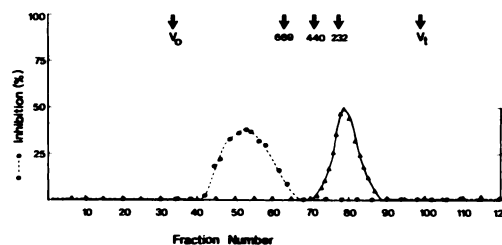


Fig 6. ACA 22 gel filtration on the supernatant of stimulated platelets. Platelets (1.15×10^8 /mL) were activated in the presence of 3 mmol/L CaCl_2 with 1.2 nmol/L thrombin and 10 μg /mL collagen at 37 °C for 15 minutes in a total volume of 32.5 mL HEPES buffer (pH 7.5) containing 0.175 mg/mL human serum albumin. The supernatant was obtained by centrifugation for 20 minutes at 15,000 rpm in a Beckman J21 B and subsequently concentrated eightfold on a PM 10 filter (Amicon, Lexington, Mass). Two milliliters were applied to an ACA 22 column (2.5×85 cm) equilibrated in HEPES buffer (pH 7.5) at 4 °C. Elution was performed with the same buffer at a flow rate of 40 mL/h, and fractions of 4.5 mL were collected. Inhibitory activity was determined by incubating 450 μL of each fraction with 25 μL factor XIa (4 nmol/L) for 15 minutes at 37 °C, after which 25 μL S2366 (5.8 mmol/L) was added and the rate of absorbance change at 405 nm recorded. The percentage of inhibitory activity was calculated from the amidolytic activity determined in the absence of inhibitor taken as 100%. Calibration of the column was achieved in a separate run using dextran blue 2000 (V_0), thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and benzamidine (V_1).

Table 2. The Effect of Trypsin on the Activity of the Inhibitor in Factor IX Activation by Factor XIa

Activator	Rate of Factor IX Activation (pmol IXa/min/mL)
Factor XIa	3.97
Factor XIa, inhibitor	0.61
Factor XIa, inhibitor after incubation with Sepharose 4B	0.63
Factor XIa, inhibitor after incubation with trypsin-Sepharose	4.06

Human factor IX was activated by human factor XIa at 37 °C in HEPES buffer (pH 7.5) containing 100 nmol/L factor IX, 0.1 nmol/L factor XIa, 0.5 mg/mL human serum albumin, and 10 mmol/L CaCl₂ in the absence or presence of inhibitor. The rate of factor IXa formation was determined as described in Materials and Methods. Factor XIa was preincubated with inhibitor for 15 minutes at 37 °C before addition to the reaction mixture. The effect of trypsin on the inhibitor was determined by incubating 1.0 mL inhibitor with 0.25 mL trypsin-Sepharose slurry (or Sepharose 4B as a control) at 4 °C. After two hours, the suspension was centrifuged and the supernatant added to factor XIa to determine the residual inhibitory activity. The inhibitor preparation used was obtained from the ACA-22 column shown in Fig 6. Fractions 48 to 58 of this column contained inhibitory activity and were pooled. Human serum albumin, 2.5 mg, was added to the pool (44 mL), which was subsequently concentrated tenfold before use in the experiment described here.

in factor IX activation. The experiment in Fig 7 also illustrates this point. Here the time course of the disappearance of factor XIa amidolytic activity towards the chromogenic substrate S2366 was followed in an incubation mixture containing 0.4 nmol/L factor XIa and the supernatant of 2.2×10^8 /mL activated platelets (closed squares). After equilibrium was reached, part of this mixture was diluted fivefold, and the remaining activity (expressed as a percentage of the amount of factor XIa present) was followed with time (closed circles). As can be seen, the amidolytic activity increased in the diluted mixture until the same final level was found as in a mixture that had been diluted fivefold at time 0

Table 3. Effect of Dilution on the Activity of the Inhibitor in Factor IX Activation by Factor XIa

Activator	Rate of Factor IXa Formation (pmol IXa/min/mL)	
	Before Dilution	After Dilution
Factor XIa (0.1 nmol/L)	3.77	0.69
Factor XIa (0.1 nmol/L), supernatant of 2×10^8 /mL platelets	0.80	0.49

Factor XIa (0.11 nmol/L) was incubated at 37 °C in HEPES buffer (pH 7.5) containing 1 mg/mL human serum albumin and 10 mmol/L CaCl₂ in the presence or absence of supernatant from 2.2×10^8 /mL stimulated platelets (obtained as described in the legend to Fig 6). After 15 minutes, part of this mixture was diluted fivefold in the same buffer and further incubated at 37 °C for 30 minutes to allow reequilibration of factor XIa with inhibitor (see legend to Fig 7). After this time interval, factor IX activation in each of these mixtures was determined by the addition of factor IX so as to result in a final concentration of 100 nmol/L. Final concentrations of factor XIa and platelet supernatants reached are indicated in the table and were fivefold less in the diluted mixtures. The rate of factor IXa formation was then determined by withdrawing samples from these reaction mixtures at two and four minutes and assaying for factor IXa as described in Materials and Methods.

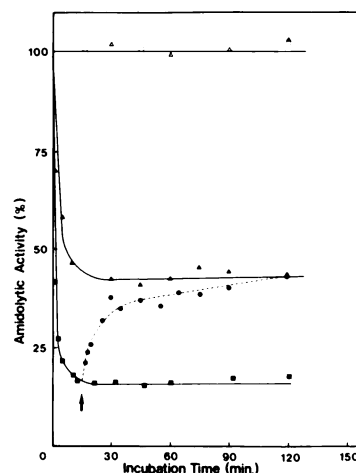


Fig 7. Reversibility of factor XIa inhibition. The supernatant (7.65 mL) of 2.6×10^8 /mL platelets (obtained as described in the legend to Fig 5) was incubated for five minutes at 37 °C, and 0.9 mL factor XIa (4 nmol/L) was added. Immediately thereafter, 1.5 mL was subsampled and diluted fivefold in HEPES buffer containing 0.5 mg/mL human serum albumin. The time course of inhibition in each reaction mixture was followed by subsampling 475 μ L to a cuvette at the time intervals indicated in the figure. Twenty-five microliters of S2366 (5.8 mmol/L) were added, and the rate of change of absorbance at 405 to 500 nm was determined at 37 °C on a DW2A Spectrophotometer (Amicon, Lexington, Mass). As a control, the amidolytic activity was followed over time in two reaction mixtures (prepared the same way) without supernatant present. The final concentrations in each reaction mixture were 0.5 mg/mL human serum albumin in HEPES buffer (pH 7.5); factor XIa (0.4 nmol/L), no platelet supernatant (□); factor XIa (0.4 nmol/L) and the supernatant of 2.2×10^8 /mL platelets (■); factor XIa (0.08 nmol/L), no platelet supernatant (△); and factor XIa (0.08 nmol/L) and the supernatant of 4.4×10^7 /mL platelets (▲). After equilibrium was reached, reversibility of the inhibition was verified by diluting part of the mixture containing the high concentrations of reactants fivefold and following the reappearance of amidolytic activity (●). The amidolytic activities determined for each reaction mixture in the presence of platelet supernatant were expressed as a percentage of the activities determined in the reaction mixture containing corresponding amounts of factor XIa but without supernatant present (open symbols).

(closed triangles). Factor XIa activity was stable throughout the time course of the experiment in both the diluted and undiluted mixtures in the absence of platelet supernatant (open symbols). These data indicate that the inhibition of factor XIa was completely reversible.

Figure 8 shows Lineweaver-Burk plots of factor IX activation by factor XIa in the presence of varying amounts of platelet supernatant. These data show that both V_{max} and K_m were affected, indicating that the inhibitor is of the mixed type.

DISCUSSION

In this paper we report the effects of stimulated human platelets on the activation of human factor IX by factor XIa. We developed an assay that measures functional factor IXa via its ability to activate bovine factor X as described earlier¹⁴ except that apart from phospholipids and CaCl₂ bovine factor VIIIa was also present in the assay. The

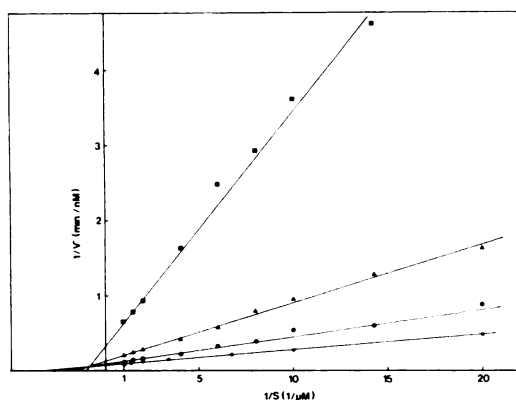


Fig 8. Lineweaver-Burk plots of factor IX activation by factor XIa at various inhibitor concentrations. The kinetic parameters (K_m for factor IX and V_{max}) were determined in the presence of varying amounts of inhibitor. Variation in the inhibitor concentrations was obtained by stimulating different platelet concentrations for twelve minutes at 37 °C in the presence of 3 mmol/L CaCl_2 with thrombin (1.2 nmol/L) and collagen (10 $\mu\text{g}/\text{mL}$) under continuous stirring followed by centrifugation for two minutes in an Eppendorf Microfuge to obtain the supernatants. Subsequently, the CaCl_2 concentration was adjusted to 10 mmol/L, and factor XIa was added and incubated for ten minutes at 37 °C before the addition of factor IX. The final reaction mixtures contained 10 mmol/L CaCl_2 , 0.5 mg/mL human serum albumin, varying amounts of factor IX, and 0.1 nmol/L factor XIa in HEPES buffer (pH 7.5) without inhibitor (○) and in the presence of supernatant obtained from $2 \times 10^7/\text{mL}$ platelets (●), $6 \times 10^7/\text{mL}$ platelets (▲), and $2 \times 10^8/\text{mL}$ platelets (■). Rates of factor IX activation were determined as described in Materials and Methods, and from these, the Lineweaver-Burk plots were constructed. The kinetic parameters obtained were 14.4 nmol/L factor IXa/min and 0.3 $\mu\text{mol}/\text{L}$ (○), 10.0 nmol/L factor IXa/min and 0.37 $\mu\text{mol}/\text{L}$ (●), 7.9 nmol/L factor IXa/min and 0.6 $\mu\text{mol}/\text{L}$ (▲), and 3.0 nmol/L factor IXa/min and 0.99 $\mu\text{mol}/\text{L}$ (■).

presence of factor VIIIa increases the sensitivity some 3,000-fold but has the disadvantage that for each new batch of factor VIII a new standard curve must be made. When this is done, a good day-to-day reproducibility can be achieved, and the assay allows accurate determination of factor IXa down to levels of 2.5 pmol/mL (Fig 1). Different batches of factor IXa, factor X, and phospholipid can be used interchangeably to produce the same standard curve provided equal amounts of these reagents are used. Using this assay, we determined the kinetic parameters of factor IX activation by factor XIa, and we found a K_m for factor IX of 0.3 $\mu\text{mol}/\text{L}$ and a k_{cat} of 2.4 s^{-1} /factor XIa molecule of 160,000 mol wt. Some disagreement exists in the literature concerning these kinetic parameters. Walsh et al¹⁰ using factor XIa activated with bovine factor XIIa found a K_m of 0.49 $\mu\text{mol}/\text{L}$ and a k_{cat} of 7.7 s^{-1} , whereas Bajaj¹³ using a trypsin-activated factor XIa found a k_m of 2.0 $\mu\text{mol}/\text{L}$ and a k_{cat} of 0.34 s^{-1} (per 160,000 mol wt factor XIa). Both these studies made use of the tritiated activation peptide release. The kinetic parameters determined by us are rather close to the data reported by Walsh et al.¹⁰

Using this assay, we determined the effect of platelets on the rate of factor IXa formation by factor XIa. The data reported here clearly demonstrate that stimulated platelets

cause a dramatic decrease in the rate of factor IX activation by factor XIa, whereas unstimulated platelets have no effect. This inhibition is caused by a direct interaction with factor XIa, since steady-state rates of factor IXa formation can only be achieved by incubation of factor XIa with the stimulated platelets, whereas preincubation of factor IX has no effect (Fig 2). Moreover, factor XIa amidolytic activity towards the chromogenic substrate S2366 is also inhibited (see Figs 6 and 7). Various platelet agonists can bring about this effect, the most potent being thrombin or the ionophore A23187. The set of agonists that can induce this effect (Table 1) as well as the rapid time course of the appearance of inhibitory activity after stimulation of the platelets (Fig 5) suggests the involvement of a release reaction. This is in accordance with the finding that the inhibitory activity is quantitatively recovered in the supernatant after centrifugation in a microfuge for five minutes (Fig 3). The amount of inhibitory activity is dependent on the amounts of stimulated platelets present. Thus, under the conditions used in our experiments, factor IX activation is not affected by the presence of $10^6/\text{mL}$ platelets. At the physiologic platelet concentration ($2 \times 10^8/\text{mL}$), about 10% of the factor IX activation rate is left. Thus, the inhibitory activity reported here may play a role under physiologic circumstances. However, more experiments will be needed to gain insight into this.

The high apparent mol wt with which the inhibitor elutes from an ACA 22 column as well as the rather broad elution profile (see Fig 6) suggests that the inhibitor consists or is part of high-mol wt complexes. This may indicate that the inhibitor is associated with the so-called platelet dust.³³ However, an essential part of the large inhibitor molecules eluting from the ACA 22 column must be proteinlike in nature, since the inhibitory activity was completely destroyed after tryptic digestion. Moreover, the activity is also destroyed upon boiling. Therefore, at this moment we work under the hypothesis that the inhibitory activity is due to a protein that is released. This is most likely an antiprotease that reversibly blocks the factor XIa active sites both for factor IX activation and for S2366 conversion, since the inhibition seems reversible (see Table 3 and Fig 6). This inhibition is of the mixed type as determined by Lineweaver-Burk analysis of factor IX activation by factor XIa in the presence of varying amounts of inhibitor (Fig 8).

The main plasma inhibitors of factor XIa are α_1 -antitrypsin and antithrombin III.³⁴ α_1 -Antitrypsin is also present in platelets and can be secreted upon stimulation.³⁵ However, these inhibitors have a much lower molecular weight and irreversibly block factor XIa. Therefore, our data do not suggest any involvement of these proteins in the inhibition that we observe in our experiments. At this moment we do not know whether under different experimental conditions the α_1 -antitrypsin present in platelets may become involved in the inhibition of factor IX activation by factor XIa.

It has been suggested that high-mol wt kinogen (HMWK) protects factor XIa against inactivation by plasma inhibitors,³⁴ and a recent report of Schmaier et al shows that HMWK can be secreted from human platelets

upon stimulation.³⁶ The results presented here show that despite these amounts of HMWK present in the platelets the rate of factor IX activation by factor XIa decreases in the presence of stimulated platelets. Sinha et al have recently presented evidence that factor XIa needs HMWK to bind to stimulated platelets and that the amount of HMWK secreted by platelets is not sufficient to cause measureable factor XIa binding to triggered platelets.³⁷ Therefore, it will be important to investigate whether factor XIa binding via HMWK to

stimulated platelets can prevent the inhibition of factor IXa formation caused by stimulated platelets in the absence of external HMWK.

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